Peer Review File

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Reviewer A

Comment 1: I have noticed several typing errors throughout the manuscript that may be edited either directly by the author or by a native English speaker preferentially. Also, it would be important to strictly follow the nomenclature rules: for example, allele must be in italics, such as in “DEL allele”, “weak D alleles”, “RHD*01EL.01”, “RHD*04.01”... It would be better to replace “D positive” by “D-positive” (and then “D+” once defined), the same to be used for “D negative”. Prefer “D-negative” to “RhD-negative”.

Reply 1: I thank the reviewer for his observation and apologize for these inconsistencies. The manuscript was checked for typing errors and the nomenclature was checked and adapted.

Changes in the text: Throughout the manuscript, allele and genes are now written in italics. D positive and D negative have been replaced by D-positive and D-negative in the first occurrences and then by D+ and D-. RhD-negative was replaced by D- or by RHD-negative depending on whether the focus was on the absence of the D antigen or the absence of the RHD gene.

In addition, line 202/203 “In three alleles of these alleles” was changed to “In three of these alleles”

Comment 2: In terms of the structure of the manuscript, I would recommend reorganizing as indicated below by using subheadings for clarity:

I. Introduction

II. The importance of Del

III. The Asian Del Phenotype

IV. The difficulties to discriminate the Del phenotype from other D phenotypes

1. Del or partial D?

2. Del or weak D?

3. Antigen density of Del

4. Del or D-negative (D–)?

V. The molecular bases of Del

1. DEL alleles with missense mutations

2. DEL alleles with splice site mutations

3. DEL alleles with a hybrid structure

4. DEL alleles with frameshift mutations

5. DEL alleles with premature termination codons

6. DEL alleles with major alterations in the RH genes including deletions of whole

exons or loss of start or stop codon

7. DEL alleles with missense mutations

VI. Worldwide distribution of Del VII. Anti-D immunization

1. In Del patients

2. By RBC units from Del donors

VIII. Conclusion

Reply 2: The same issue was raised by the editor (editorial comment 4-3) who suggested that a slightly different type of numbering. For consistency purposes, I stuck with the editor's suggestion.

Changes in the text: All subheadings were numbered using the numbering type suggested by the editor. A new subheading "anti-D immunization" was introduced before "anti-D immunization in Del patients".

Comment 3: l.84: Minor allele is "C" not "T". Thus the variant should be "c.1073+923T>C". Please also indicate the rs number (i.e. rs2427766) for this variant, as well as for c.1073+152C>A (i.e. rs41307824).

Reply 3: The reviewer is fully correct, both the nucleotide present in the reference sequence and the frequent nucleotide at this SNV are "T", while "C" is the minor allele. Since Liu really described a C>T substitution (i.e. the presence of the reference sequence nucleotide), I had to rewrite the sentence.

Changes in the text: The rs numbers were added. The c.1073+923C>T substitution described by Liu was put into quotation marks. The sentence indicating the frequency of the mutation was corrected to: "c.1073+923T is the major allele at this SNV position among RHD alleles with a worldwide prevalence of 87% according to gnomAD"

Comment 4: l.203: The term "diagnostic" here is unclear. Please clarify.

Reply 4: The ISBT table list "differences" from normal RHD as characteristic for these DEL alleles. I admit that "diagnostic" might not clearly describe this situation and therefore replaced the description with a slightly different wording.

Changes in the text: ""diagnostic" polymorphism" indicated was replaced by "the polymorphism indicated in the ISBT table as difference to normal RHD"

Comment 5: l.228 to 245: Because impact of the missense variants on protein structure is very likely to be more frequent than splicing disruption, it would make more sense to indicate it first, that is inverting the mechanisms in the text.

Reply 5: The order of the paragraphs represents the order of interference with protein synthesis: First, the RNA is spliced, afterwards, the mRNA is translated. Inverting the order of the paragraphs has the additional disadvantage that the second mechanism would come after a long explanation of the impact of missense mutations. However, I clarified that the order does not reflect the importance of the mechanisms

Changes in the text: the words “two non-exclusive mechanisms” were supplemented with the clarification “(listed in the order of interference with protein synthesis)”. The following paragraph on the splicing mechanism was introduced with “sometimes” clarifying that the mechanism is not the major mechanism.

Comment 6: l.233-234: I agree that a deleterious effect on protein structure “may interfere with protein integration into the membrane”. But it may also affect other mechanisms, such as protein folding, intramolecular interactions, intermolecular interactions with partners... It would be worthwhile to be stated here.

Reply 6: I believed that reduced integration into the membrane would be the common endpoint of the mechanisms mentioned by the reviewer. However, I have to admit that there are no data proving this assumption and alternatively these mechanisms could abolish D antigen expression without impacting on protein integration. Therefore, I rewrote the sentence..

Changes in the text: the mechanisms suggested have been added as “and may disrupt protein folding, intramolecular interactions, and intermolecular interactions with partners leading to reduced protein integration into the membrane or antigen loss.”

Comment 7: l.238: It is unclear to me why the author “chose” to analyse the first 27 weak D alleles. In clear, why 27? What is the rationale?

Reply 7: The number of weak D alleles analyzed was identical to the number of DEL alleles analyzed (23 alleles). Since type 4, 11, and 15 are not considered weak D by ISBT and type 14 is not due to single missense mutations, the 23st weak D allele according to the ISBT listing with a single missense mutation is weak D type 27

Changes in the text: The reason for weak D type27 is explained now: “the 23 DEL alleles were compared with the same number of weak D alleles, which covers the range weak D type 1 to type 27, because type 4, type 11 and type 15 are not included in the ISBT weak D list and type 14 is not due to a single SNV”

Comment 8: l.257-259: As indicated above, adsorption-elution test may yield false-negative, but also false- positive results. On that basis, the reported effect of specific variants, such as c.486+1G>A involved in the RHD*01EL.08 allele assumed to cause a partial Del phenotype, may be challenged. Indeed, unless I am wrong, from a molecular point of view, reference #97 does not seem to support a "19 amino acid insertion after Asn162" as indicated in Table 3. Another possible explanation may likely be that this allele causes a D-negative phenotype. Please explain and/or reference.

Reply 8: I have to admit that reference 97 is not the correct reference (throughout the manuscript), because the wrong manuscript of M. Christiansen was referenced. The correct reference is Christiansen M, Sorensen BM, Grunnet N, RHD positive among C/E+ and D- blood donors in Denmark, TRANSFUSION 2010;50:1460-1464 now included as reference 63. In table 1 of this reference, the insertion hypothesis is given as footnote. The Del phenotype of RHD*01EL.08 was reported in the initial publication (ref 20), independently confirmed by Körmöczi (ref 43) using adsorption/ elution and also by Single molecule fluorescence microscopy complemented by machine learning (ref 44).

Changes in the text: The reference was corrected.

Comment 9: l.277-279: I definitely agree that "analysis of mRNA and minigene splicing assays... cannot replace serology yet". But it is worthwhile adding "as far as serological data are solid and confirmed by independent observations".

Reply 9: I agree with the reviewer's feeling that some serologic phenotype descriptions are most likely incorrect. However, my interpretation is slightly different: If the molecular predictions may be incorrect, they may be incorrect. This statement is independent from the statement that some serologic descriptions are most likely incorrect. Personally, I believe it is too early to drop serology. Still, molecular predictions are very helpful to identify cases in which the serology needs reevaluation. Therefore, I added the phrase suggested by the reviewer in a slightly modified form. I hope he can agree.

Changes in the text: A phrase was added: "It is important that serological data are solid and confirmed by independent observations, especially if they are discrepant to the phenotype expected based on molecular studies."

Comment 10: l.351-353: Unclear. The author seems to be talking about NL-3 (i.e. RHCE(1-8)-D(9-10)) rather than NL-6 (i.e. RHD(1-9)-RHCE(10)). But the final part of the sentence does not fit well with what indicated before. Please check and modify accordingly.

Reply 10: This paragraph was indeed on NL-3, not on NL-6. The final part of the sentence has been revised to make it more comprehensible.

Changes in the text: NL-6 was corrected to NL-3. The final sentence was revised: "The origin of the D antigen in this haplotype is unknown: RhD(1-7) seems to be unexpressed. RhCE and RhCE-D(9 to 10) differ by a single amino acid located in the C-terminal intracellular protein segment and it is difficult to imagine how such change can lead to antigen D expression."

Comment 11: l.413: Please indicate in which patients? D-negative, weak D, partial D?

Reply 11: As the heading seems to be not clear enough, now I indicate that the patients are D-

Changes in the text: The subheading was changed to “Anti-D immunization of D- patients by RBC units from Del donors”

Minor Comment 1: l.74: Replace “strangely enough” by “curiously”.

Reply M1: I thank for this suggestion

Changes in the text: The wording was changed to curiously as suggested.

Minor Comment 2: l.75: Use “synonymous” rather than “silent” to qualify the c.1227G>A substitution, because the latter suggests that this variant has no effect while the former indicates that the sequence of the protein is not modified, which is slightly different from a functional point of view.

Reply M2: I thank for this suggestion

Changes in the text: The wording was changed to synonymous as suggested

Minor Comment 3: l.88: Replace “RHD*EL1.01” by “RHD*01EL.01”.

Reply M3: I thank for this suggestion

Changes in the text: The spelling was changed to RHD*01EL.01 as suggested

Minor Comment 4: l.156: Remove “than”.

Reply M4: I thank for this suggestion I selected the wrong word when I tried to describe a temporal relationship.

Changes in the text: Than was replaced by “later” to indicate the temporal scale.

Minor Comment 5: l.221: Remove “in” that is repeated several times. Replace “are” by “have been”.

Reply M5: I thank for this suggestion.

Changes in the text: The superfluous “in”s were removed, are was replaced by “have been”. The sentence now reads: “The alterations described in three alleles cannot be the cause of a Del phenotype and there have been repeated observations (26, 55, 67, 100) of a Del phenotype in

samples with a seemingly normal RHD gene indicating that some causes of DEL cannot be found by currently used methods."

Minor Comment 6: l263: I guess transcripts lacking RHD exon 9 encode for a 393 amino acid protein, not a 463 amino acid protein. Please check and modify if necessary.

Reply M6: The deletion of RHD exon 9 leads to a frameshift and the stop codon is missed. Therefore, the protein is elongated to 463 amino acids.

Changes in the text: No changes.

Minor Comment 7: l.376: Add "%" after "0.03" and "0.28".

Reply M7: I thank for this suggestion

Changes in the text: The "%" symbols were added

Minor Comment 8: l.391: It would be better to use the recommended nomenclature rather than RHCE*ce(W16C, A36T, L245V) for homogeneity. So please mention RHCE*01.20.13 or RHCE*ce.20.13 or RHCE*ceVS.13 (www.isbtweb.org/resource/004rhce.html or www.bloodgroupgenomics.org/rhce/rhce-table/).

Reply M8: I thank for this suggestion

Changes in the text: RHCE*ce(W16C, A36T, L245V) was changed to RHCE*ce.20.13

Reviewer B

Comment 1: Del is now consistently written as DEL. This should be updated (line 21 and throughout). DEL is used in the title but rarely elsewhere: was there a reason? If Del is mentioned at all, the transition and its reason should be explained along when it occurred.

Reply 1: In this review, the abbreviations used were based on the AABB conventions: AABB consistently uses Del for the phenotype and DEL for the allele. A statement indicating this fact has been added in the introduction. In the title, I used DEL because the molecular biology relates to alleles. Concerning Asian-type DEL, the reviewer is undoubtedly correct, because the Asian-type DEL is a molecular diagnosis. Therefore, Asian-type Del was changed to Asian-type DEL throughout the manuscript.

Changes in the text: An explanatory statement was added in the introduction: "(in line with AABB suggestions, in this review Del is used for the phenotype and DEL for the allele)". Throughout, the

manuscript was carefully checked whether DEL/Del related to an allele or a phenotype and the writing adjusted if necessary. Asian-type DEL is now written as “Asian-type DEL” throughout.

Comment 2: For instance in line 66, it would be unfortunate if an incorrect, not previously defined term “Asian Del” was used as headline.

Reply 2 Concerning Asian-type DEL, the reviewer is undoubtedly correct, because the Asian-type DEL is a molecular diagnosis. Therefore, Asian-type Del was changed to Asian-type DEL throughout the manuscript.

Changes in the text: Asian-type Del was corrected to “Asian-type DEL” throughout the manuscript.

Comment 3: Line 23: East Asian

Reply 3: I thank the reviewer for detecting this error

Changes in the text: East was corrected to East Asian

Comment 4: Line 25: immunization occurs in patients, not in “some samples”.

Reply 4: The reviewer has a point. However, the focus was on the alleles.

Changes in the text: The wording is now corrected to in patients carrying some alleles

Comment 5: Line 26: “mutations” should probably be changed to variants most of the time (such as lines 224, 225, 226, 251 and many other spots).

Reply 5: The reviewer is correct, the current nomenclature uses “variant” rather than mutation.

Changes in the text: All occurrences of “mutation” were replaced by “variant”

Comment 6: Line 38: delete “RBCs of”

Reply 6: I thank the reviewer for this suggestion

Changes in the text: “RBCs of” was deleted.

Comment 7: Line 42, consider: Almost 40 years later, the interest in DEL remains current because red cell genotyping enables a precision medicine approach for patients and donors with DEL variants

Reply 7: I admit that the reviewer much more precisely described what I intended to say.

Changes in the text: The old wording “Almost 40 years later, there is ongoing interest in the Del phenotype” was replaced by the reviewer’s suggestion.

Comment 8: Line 43: 7 references (3-9) aren’t that “numerous” for 40 years’ worth of research. Rephrase or add the 100+ references that apply here.

Reply 8: The seven references are key publications with review character. As I did not want to dilute this referencing with all minor comments, the sentence was rephrased.

Changes in the text: “Numerous reviews and comments on the topic” was rephrased to “Several commentaries and reviews on the topic”.

Comment 9: Line 64-65, you probably meant: “Is it possible … with sufficient reliability based on molecular data alone?

Reply 9: The reviewer has a point.

Changes in the text: The sentence was changed to “? Is it possible to predict a Del phenotype with sufficient reliability based on molecular data alone in a proband who carries a previously unknown allele?”

Comment 10: Line 75: The authors of ref. 20 are too humble. This study is fundamental to DEL at the molecular level and deserves to be featured prominently as the basis for all subsequent DEL molecular studies. If not expanded here, ref. 20 would be an ideal jump start for the paragraph starting in line 200.

Reply 10: The reviewer is correct, in ref 20 both the major molecular mechanisms leading to DEL and the most frequent DEL allele were described for the first time. The reference is now featured as start for the paragraph on the molecular bases of DEL.

Changes in the text: As suggested, a small paragraph detailing the relevance of ref 20 for the eludication of the molecular bases of DEL alleles was added at the paragraph previously starting at line 200

Comment 11: The Asian-type DEL was originally labeled RHD(K409K), a terminology that did not catch on, but should be mentioned early on to set the terminology straight (for instance, line 76)

Reply 11: The reviewer is correct, the RHD(K409K) is now mentioned.

Changes in the text: The sentence on line 76 was changed to “This allele was first dubbed RHD(K409K), later RHD(1227G>A) and is now often referred to as “Asia type” (19) or “Asian-type” (5) DEL.”

Comment 12: Line 82, consider: SNVs

Reply 12: I thank for this suggestion

Changes in the text: SNV was changed to SNVs

Comment 13: Line 88, consider: RHD*01EL.01?

Reply 13: An embarrassing error although identified by Reviewer A (see Reply M3)

Changes in the text: The spelling was changed to RHD*01EL.01 as suggested.

Comment 14: Line 99, this proposal had been discussed at conferences a long before ref. 19 in 2010; as good ideas often have many sources, consider referencing: “They [DEL recipients] might not even develop anti-D after exposure to regular D+ RBCs …” on page 1064 of Transfusion volume 46 in 2006 as the perhaps earliest hint to this idea in print

Reply 14: I thank the reviewer for pinpointing to this reasoning hidden in a correspondence on immunization by DEL blood units. I have

Changes in the text: A reference to the source was included and the whole sentence was reworded to “After several years of discussion among the experts (36), Shao CP (19) suggested a D+ transfusion strategy”

Comment 15: Line 108: this is an interesting and correct thought. Full disclosure would admit that the expected frequency is very rare (in all populations explored so far). And approximate estimates are possible for some, for instance, European populations. Add such data to your proposal.

Reply 15: The fact is not relevant to European, but to East Asian populations where RHD*01EL.01 is frequent and weak D types 1 to 3 are rare. I have included a short calculation. However, I agree that the impact is much lower than the impact of changing the transfusion strategy for seemingly D-.

Changes in the text: A sentence was added: “While the logic of this policy is self-explanatory, the impact is limited: weak D and partial D probands are much rarer in China than individuals D- by routine serology (e.g. Yan et al. (39) observed in their donor cohort 1401 D- donors but only 37 donors with weak D or partial D). Furthermore, the likelihood of the presence of RHD*01EL.01 in weak D and partial D carriers is only about half of the likelihood in a seemingly D- individual.”

Comment 16: Line 112 (“becomes”), consider: seems possible. Not proven for most partial D, which is an often ignored, yet clincally important, technical fact.

Reply 16: I thank the reviewer for this suggestion.

Changes in the text: The word “becomes” was replaced by “seems”

Comment 17: Lines 115-116: add 2 references for the original descriptions of the alleles in parentheses

Reply 17: Considering references, I have to admit that I did not select the best examples. Therefore, RHD*04.01 was substituted by RHD*03.04 with a very high antigen density determined by flow cytometry in the first publication. Likewise, RHD*06.01 was substituted by RHD*06.02, because the first description of the molecular structure of RHD*06.01 was incorrect and no suitable citation.

Changes in the text: RHD*04.01 was changed to RHD*03.04, RHD*06.01 was changed to RHD*06.02 and references were added.

Comment 18: “partial Del” (line 116 and numerous times elsewhere) seems not well defined, and I would propose a more descriptive term, such as “DEL with lack of epD” or “DEL lacking some epD”. This technical approach is generally applied for the entity and circumvents the need to rely on anti-D occurrences.

Reply 18: The term “partial DEL” was first used by Körmöczi in 2005 (ref 43). In my view, it is as defined respectively not defined as the term “partial D”. The term is used throughout on purpose to pinpoint to the difficulties to prove / exclude a partial D phenomenon in a Del phenotype (which technically may be impossible, as both discrimination of alloantibodies from autoantibodies and the demonstration of the presence / absence of a D epitope may be impossible in a Del phenotype.

Changes in the text: A sentence was added clarifying my position. “While this concept seems intuitive at first glance, the verification that a Del phenotype expresses a partial D antigen may be painstakingly difficult and sometimes impossible”

Comment 19: Line 121: This correct claim should be supported by one or more references.

Reply 19: I thank for the suggestion

Changes in the text: A reference was added (ref 43)

Comment 20: Line 137: “several confounding factor” remains vague to the nature of the problem. Consider: technical details that preclude a simple distinction

Reply 20: I thank the reviewer for the suggestion

Changes in the text: The phrase was revised to “the result heavily depends on technical details precluding a simple distinction:”

Comment 21: Line 145, consider: moved from … to … and on to …

Reply 21: I thank the reviewer for the suggestion

Changes in the text: The wording was changed as suggested

Comment 22: Lines 156-157, consider: “thEn observed … and later was listed as partial D by ISBT.”

Reply 22: I thank the reviewer for the suggestion

Changes in the text: The wording was changed as suggested

Comment 23: Line 160, consider finishing this sentence by: …, which is scientifically untenable and clinically misleading.

Reply 23: I think pinpointing to these examples is explicit enough. Most people will realize that it cannot be the aim of a nomenclature to invent two names for one allele suggesting two different phenotypes. However, it is not the fault of the person responsible for RHD, it is a consequence of the decision of the working party to use a “phenotype first” numbering for the alleles.

Changes in the text: I would like to keep this point as it is.

Comment 24: Line 173, add: a “monoclonal” anti-D. The test will be negative, not really “false-negative”

Reply 24: The reviewer is correct, the test is negative, not false-negative, but the interpretation is incorrect. I tried to describe the problem in more detail.

Changes in the text: The sentence now reads “In a partial Del, use of a monoclonal anti-D directed to D epitopes absent in the partial Del type will result in a negative adsorption/elution test and the Del status may be missed if only this anti-D is used.”

Comment 25: Line 182-183, consider: Many blood grouping instruments, such as the PK series (Olympus, give details)

Reply 25: This is a very theoretical discussion. I added PK 7300 and weakened the statements

Changes in the text: PK 7300 was added as example and the wording was weakened (using might and would)

Comment 26: Line 184, consider: “A contamination of D negative RBC by 0.1% D positive”

Reply 26: I thank the reviewer for the suggestion

Changes in the text The wording was changed as suggested

Comment 27: Lines 190-191: Add at least 1 reference for each claim to “frameshift variations” and “whole exon deletions”

Reply 27: As suggested, I added references. I chose DEL18 and DEL30 as these are among the best documented examples.

Changes in the text: References were added

Comment 28: Line 203, delete the first “alleles”.

Reply 28: I followed the recommendation.

Changes in the text: The duplicated “alleles” was deleted.

Comment 29: Line 221, in this 1 line you ought to delete “in” twice!

Reply 29: I apologize for this error

Changes in the text: The wrong “in”s were deleted

Comment 30: Line 247: this “correct” is dispensable and should be deleted

Reply 30: The reviewer is correct.

Changes in the text: The dispensable “correct” was deleted. The phrase now reads “The variations in many Del including the Asian-type DEL RHD*01EL.01 interfere with splicing.”

Comment 31: Line 410: the “non-partial” DEL phenotype shows the problem with the term “partial Del”. Consider: “the expression of all clinically relevant epD by a given DEL allele must be based …”

Reply 31: I understand that “non-partial” is a problematic term. However, the kind suggestion of the reviewer does not exactly express my position. Therefore, I used a different rephrasing

Changes in the text: The sentence now starts as “Hence, the absence of an anti-D immunization risk in carriers of a specific DEL allele must be based…”

Comment 32: Line 412, consider finishing this sentence by: … although the database is expected to vastly improve over time for the Asian-type and many other DEL alleles.

Reply 32: I thank for this suggestion

Changes in the text: The phrase was added.

Comment 33: Line 416, “immediately”: give evidence for your claim. It all started no later than ref. 20

Reply 33: I do not really understand the argument. The issue was considered in the ref 20 which is “immediately” for me.

Changes in the text: A clarification was added “and a look back study was included in this publication”.

Comment 34: Lines 452 (population) and 466 (States): add a reference each.

Reply 34: References were added

Changes in the text: The references were added

Comment 35: Line 470: Implementations in Switzerland and Germany have been claimed to be cost neutral. Consider adding a statement with references

Reply 35: A reference to a review on the use of RHD PCR for DEL testing was added

Changes in the text: The reference was added.

Comment 36: Recommend substantial re-writing of the Conclusion paragraph (start at line 485) and then adding its gist to the Abstract (which is currently a bit short)

Reply 36: Most likely, the reviewer is correct

Changes in the text: The abstract and the conclusion paragraph were rewritten